(12) UK Patent Application (19) GB (11) 2 177 914 A

(43) Application published 4 Feb 1987

(21) Application No 8613320

(22) Date of filing 2 Jun 1986

(30) Priority data

(31) 60/121126 (32) 4 Jun 1985 (33) JP

(71) Applicant
Chugai Seiyaku Kabushiki Kaisha

(Incorporated in Japan),

5—1 5-chome Ukima, Kita-ku, Tokyo, Japan

(72) Inventors
Naoto Shimoda,
Kazuo Igusa

(74) Agent and/or Address for Service
Haseltine Lake & Co., Hazlitt House, 28 Southampton Buildings, Chancery Lane, London WC2A 1AT

(51) INT CL⁴
A61K 37/02 9/00

(52) Domestic classification (Edition I)
A5B 170 316 31X 31Y 342 343 34Y 822 823 824 826 H L
U1S 1313 A5B

(56) Documents cited
GB A 2171304
GB A 2171303
GB A 2135676
EP A2 0148605
Merck Index-10th Edition, page 533

(58) Field of search
A5B
Selected US specifications from IPC sub-class A61K

(54) Erythropoietin compositions

(57) Nasal composition contain human erythropoietin for the treatment of anemia. Preferred formulations comprise an aqueous and/or non-aqueous medium containing human erythropoietin and a surface active agent.

GB 2 177 914 A

SPECIFICATION

A Pharmaceutical Composition for Nasal Administration for the Treatment of Anemia

The present invention relates to pharmaceutical composition for nasal administration for the treatment of anemia.

Human erythropoietin (hereinafter sometimes abbreviated as human EPO) is a glycoprotein which is principally produced in the kidney and is known as a humoral hemopoietic factor that acts on erythroblastic stem cells in the bone marrow to promote their differentiation into erythrocytic cells. Human erythropoietin is considered to have great potential for use as therapeutics for the treatment of various types of anemia.

Most proteins are either sparingly absorbable or are digested and decomposed before they are absorbed in the stomach or intestines. Hence, they are usually administered by injection into blood vessels or tissues. However, injections cause much pain and inconvenience in patients who therefore have not a few complaints about treatments with injections.

As alternatives to administration by injection, methods have recently been proposed for administering proteins into the rectum, bronchia, eyes or nasal cavity. The fact that effective absorption of insulin and calcitonin was attained by nasal administration suggests its potential as a new treating method with proteins.

Human EPO is believed to belong to the same category as proteins and it is strongly desired to establish a method of administering human EPO by means other than injection. However, no case has yet been reported of successful administration of human EPO by methods other than injection.

Under these circumstances, the present inventors conducted experiments wherein human EPO was administered into the nasal cavity of animal models with anemia. To their great surprise, the human EPO so administered was absorbed in high doses and proved effective in treatment of anemia. The present invention has been accomplished on the basis of this finding.

The present invention therefore relates to the provision of a novel pharmaceutical composition for nasal administration for the treatment of anemia.

The present invention relates to novel pharmaceutical compositions for nasal administration containing human EPO as an active ingredient for the treatment of anemia. More specifically, the invention relates to novel pharmaceutical compositions for nasal administration comprising an aqueous and/or non-aqueous medium containing human EPO and a surface active agent for the treatment of anemia.

The human EPO incorporated in the pharmaceutical composition of the present invention is defined as a trace physiologically active substance that governs proliferation, differentiation and maturation of erythroblastic stem cells into erythrocytic cells. Therefore, the term "human EPO" as used herein includes not only naturally occurring forms of human EPO but also derivatives and analogues thereof which have pharmaceutical activities.

The human EPO that is incorporated in the composition of the present invention as the active ingredient may be prepared by a variety of means. For example, human EPO may be extracted from normal human urine or the urine or plasma (including serum) of patients with aplastic anemia [T. Miyake et al., J. B. C., *252*, 5558 (1977); and J. P. Lewin et al., J. Lab. Clin. Med., *66*, 987 (1965)]. Human EPO may be prepared from, for example, tissue cultures of human kidney cancer cells (Unexamined Published Japanese Patent Application No. 55790/1979), from human lymphoblastic cells having the human EPO producing ability (Unexamined Published Japanese Patent Application No. 40411/1982), and from a culture of the hybridoma obtained by cell fusion of a human cell line. Human EPO may also be prepared by genetic engineering procedures comprising obtaining a messenger RNA (mRNA) corresponding to the amino acid sequence of human EPO, preparing a recombinant DNA using the mRNA, and expressing the DNA gene in a suitable host cell such as a bacterium (e.g. *E. coli*), a yeast, or a plant or animal cell line [see, for example, Sylvia, L. H., Proc. Natl. Acad. Sci., USA, *81*, 2708 (1984)]. While various animal cell lines are available as host cells, cultured cell lines derived from humans or mammalian animals are preferred and they include COS cells, Chinese hamster ovary (CHO) cells, and mouse C-127 cells. Any of the types of human EPO that are prepared by these methods are useful in the present invention so long as they enable the proliferation of mature red blood cells having sufficient oxygen transport to be useful in the treatment of anemia in malignancy.

The level of administration of human EPO varies according to the condition of a patient. In the case of parenteral injection, preparations which contain 0.1 to 500 micrograms of human EPO, and preferably 5 to 100 micrograms, can be administered from one to seven times for a week. In the case of intranasal application, the levels of administration of human EPO are somewhat higher than those in parenteral injection.

Surface active agents used in accordance with the present invention may be those which promote the absorption of human EPO through nasal mucous membrane, that is to say, those which work as absorption promoters. Such surface active agents include: bile salts such as sodium cholate, sodium taurocholate and the like; cationics such as benzalkonium chloride, benzethonium chloride and the like; anionics such as polyoxyethylene alkyl ether sulfates (e.g. sodium polyoxyethylene lauryl ether sulfate) and alkyl sulfates (e.g. sodium lauryl sulfate); nonionics such as sorbitan fatty acid esters (e.g. sorbitan monostearate, sorbitan monooleate and sorbitan sesquioleate), glycerol ester of fatty acids (e.g. glyceryl monostearate and glyceryl monooleate), polyoxyethylene sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan

monooleate), and the like; amphoterics such as imidazolinium betaines and the like; and phospholipids such as phosphatidylcholine and the like.

The amount of such a surface active agent may usually be in the range from 0.001 to 10% w/v and preferably 0.01 to 1% w/v, the amount depending on the specific surfactant used. The amount is generally kept as low as possible since above a certain level no further enhancement of absorption can be achieved and also too high surfactant levels may cause irritation of the nasal mucosa.

Examples of the aqueous media used in accordance with the present invention include water, physiological saline solution, buffer and the like. Example of the non-aqueous media include: higher aliphatic acid esters such as ethyloleate; alcohols such as ethanol, benzyl alcohol, propylene glycol, polyethylene glycol, glycerol and the like; vegetable oils such as olive oil, sesame oil, soybean oil, camellia oil, corn oil, rape seed oil, peanut oil and the like; mineral oils such as paraffin, lanolin, petrolatum and the like; dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and the like.

Such aqueous or non-aqueous media may be used either alone or in admixture in the form of solution, suspension, emulsion or ointment, if applicable to nasal mucosa.

The preparations of the present invention may also contain other additives, such as stabilizers, adsorption preventing agents and preservatives. Examples of such stabilizers and adsorption preventing agents include: proteins such as albumins, gelatins and the like; monosaccharides such as tetroses (e.g. erythrose, threose and the like), pentoses (e.g. arabinose, xylose, ribose, lyxose, ribulose, xylulose and the like), hexoses (e.g. glucose, galactose, mannose, talose, fructose, sorbose, tagatose, psicose and the like), peptanoses, octanoses and the like; oligosaccharides such as disaccharides (e.g. maltulose, maltose, cellobiose, gentiobiose, melibiose, lactose, turanose, sophorose, trehalose, isotrehalose, sucrose, lactulose and the like), trioses (e.g. maltatriose, cellotriose, manninotriose, panose, planteose, raffinose and the like) and tetraoses (e.g. stachyose, cellotetraose, scorodose and the like); deoxysaccharides such as deoxyribose, rhamnose, fucose, quinovose, tyvelose, colitose and the like; amino sugars such as glucosamine, galactosamine, mannosamine, gulosamine, kanosamine and the like; uronic acids such as glucronic acid, galacturonic acid, mannuronic acid, lactones of such uronic acids; cyclitols such as inositol, quercitol, bornesitol and the like; sugar alcohols such as glycerol, erythritol, adonitol, arabitol, mannitol, sorbitol, galactitol, sedoheptitol, perseitol, and their derivatives such as anhydrides; polysaccharides such as dextran, cellulose and the like; inorganic salts such as sodium chloride, potassium phosphate and the like; organic salts such as citrates, acetates and the like; amino acids such as glycine, alanine and the like; sulfur-containing reducing agents such as glutathione, sodium thiosulfate and the like; and polyethyleneglycol and the like. Examples of such preservatives include benzoic acid, sodium benzoate, paraoxybenzoic acid esters, chlorobutanol, cresol, phenol and the like.

If desired, the preparations of the present invention may be formulated in the form of gel by adding gel bases such as cellulose derivatives, natural gums, vinyl polymers, acrylic acid polymers, and the like.

The preparations of the present invention may be administered to nasal cavity by means of various methods. In the case of low viscosity, the preparations of the present invention may be administered to nasal cavity by means of instillation or spraying. In the case of high viscosity, they may be administered directly from tubes or by means of an applicator device which can be inserted into nasal cavity and which contains a given amount of the preparation.

Reference Example 1:

Preparation of Human Urinary EPO

Step (1): Partial Purification from Human Urine

Urine from patients with aplastic anemia was subjected to the procedures of T. Miyake et al. [J. B. C., *252*, 5558 (1977)]; viz., 1) deionization on a Sephadex G50 column, 2) adsorption on DEAE-cellulose in a batch system, 3) precipitation with ethanol, and 4) chromatography on a DEAE-agarose column. By these procedures, a partially purified form of human urinary EPO was obtained.

The partially purified human urinary EPO was dissolved in a 0.1% trifluoroacetic acid (TFA) (Alderich Chemical Co., Inc.) solution containing 24% propanol (Wako Pure Chemical Industries, Ltd.) and the solution was subjected to purification by HPLC with Hitachi Model 638-50. Absorption in the ultraviolet range at 280 nm and 220 nm was used as an indicator.

Step (2): Reserve Phase Chromatography

The so prepared sample was loaded onto a YMC-C8 column (6 mm×30 cm, product of Yamamura Chemical Co., Ltd.) equilibrated with a 0.1% TFA solution containing 24% n-propanol, and the column was eluted with the same equilibrating solution. After the unadsorbed fractions were eluted, the concentration of n-propanol was increased to 26% for eluting the active fractions. The fractions containing the EPO activity were collected and concentrated to a volume of 0.1—0.2 ml by ultrafiltration using centricon-100 (trade name of amicon).

Step (3): High-performance Molecular Sieve Chromatography

The concentrated sample was loaded onto a TSK-G3000 SW column (7.8 mm×60 cm, product of Toyo Soda Manufacturing Co., Ltd.) equilibrated with a 0.1% TFA solution containing 26% n-propanol, and the column was eluted with the same equilibrating solution. Peaks having the EPO activity were obtained at

positions corresponding to molecular weights of 25,000—30,000. These active fractions were collected and freeze-dried. The fractions had a specific activity of about 9×10^4 units/mg.

The specific activities of the samples prepared in the respective Steps (1) to (3) are listed in Table I.

TABLE I

Step	Specific activity (U/mg)
(1) partial purification	600
(2) reverse phase chromatography	10,000
(3) high-performance molecular sieve chromatography	90,000

Assay method: In accordance with the method of N.N. Iscove et al., J. Cell. Physiol., *83*, 309 (1974).

Reference Example 2

Preparation of Human EPO Derived from CHO Cells

A plasmid incorporating the gene coding for the amino acid sequence of human EPO was expressed in the Chinese hamster ovary cells (CHO cells) to produce human EPO. The procedures employed are specifically described in Japanese Patent Application No. 281862/1984 (filed December 27, 1984), entitled "Vector harboring accessory DNA for the transformation of eucaryotic cells". A summary of the procedures is given below.

The DNA from a lambda HEPOFL 13 clone incorporating the gene coding for the amino acid sequence of human EPO derived from fetal human liver cells was digested with EcoRI, and the recovered small EcoRI fragment harboring the gene coding for the amino acid sequence of human EPO was inserted into the EcoRI site of plasmit RKI-4. The plasmid then was incorporated into DHFR-deficient CHO cells so as to transform them. The transformed CHO cells were cultured in an alpha-medium deficient of nucleic acids. Cells harboring at least one DHFR gene were selected and employed in the production of human EPO, with the concentration of methotrexate in the medium being increased incrementally. The human EPO in the supernatant of the finally obtained culture had an activity of 20 units/ml. The resulting culture solution containing human EPO was dialyzed against physiological saline solution containing 0.1% BSA and subjected to the following experiment.

The human EPOs prepared in Reference EXAMPLEs 1 and 2 were administered intranasally for a week in several doses per day to rats with Shibata nephritis and mice bearing Lewis lung carcinoma. The erythrocyte counts increased and returned to the normal levels. No significant toxicity occurred in the animals throughout the period of human EPO administration.

The following examples are provided for the purpose of further illustrating the present invention but are by no means taken as limiting.

EXAMPLE 1

	% w/v
Human EPO	0.001
Glyceryl monostearate	0.5
Dextran	1
Purified water Q.S.	100

EXAMPLE 2

	% w/v
Human EPO	0.001
Sodium cholate	0.5
Methylcellulose	1
Purified water Q.S.	100

EXAMPLE 3

	% w/v
Human EPO	0.001
Polyoxyethylene lauryl ether	0.5
Polyethylene glycol	1
Purified water Q.S.	100

EXAMPLE 4

	% w/v
Human EPO	0.001
Benzalkonium chloride	0.5
Polyethylene glycol	0.5
Purified water Q.S.	100

EXAMPLE 5

	% w/v
Human EPO	0.001
Phosphatidylcholine	0.2
Purified water Q.S.	100

EXAMPLE 6

	% w/v
Human EPO	0.001
Sodium lauryl sulfate	0.5
Methyl paraoxybenzoate	0.12
Propyl paraoxybenzoate	0.01
Methyl cellulose	1
Purified water Q.S.	100

EXAMPLE 7

	% w/v
Human EPO	0.01
Phosphatidylcholine	0.2
Sodium acetate, trihydrate	1.36
Acetic acid	0.6
Chlorobutanol	0.1
Purified water Q.S.	100

EXAMPLE 8

	% w/v
Human EPO	0.01
Sorbitan monooleate	0.02
Propylene glycol Q.S.	100

CLAIMS

1. A pharmaceutical composition for nasal administration containing human erythropoietin as an active ingredient for the treatment of anemia.

2. A pharmaceutical composition for nasal administration comprising an aqueous and/or non-aqueous medium containing human erythropoietin and a surface active agent for the treatment of anemia.

3. A pharmaceutical composition for nasal administration comprising an aqueous and/or non-aqueous medium and a gel base containing human erythropoietin and a surface active agent for the treatment of anemia.

4. For use in the treatment of anemia, human erythropoietin.

Printed for Her Majesty's Stationery Office by Courier Press, Leamington Spa. 2/1987. Demand No. 8817356.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.